Controlled *In Vitro* Delivery of Voriconazole and Diclofenac to the Cornea using Contact Lenses for the Treatment of *Acanthamoeba* Keratitis

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Abstract (limit 200)

Acanthamoeba keratitis is caused by a protozoal infection of the cornea, with 80% of cases involving the improper use of contact lenses. The infection causes intense pain and is potentially blinding. However, early diagnosis improves treatment efficacy and the chances of healing. Despite the apparent accessibility of the cornea, patients do not always respond well to current eye drop treatments largely due to rapid dose loss due to blinking and nasolacrimal drainage. Here, the topical drug delivery of voriconazole alone and in combination with diclofenac via drug-loaded contact lenses, were investigated *in vitro*. The contact lenses were applied onto excised porcine eyeballs and maintained at 32 °C under constant irrigation, with simulated tear fluid applied to mimic *in vivo* conditions. The drug delivered to the corneas was quantified by HPLC analysis. The system was further tested in terms of cytotoxicity and a scratch wound repopulation model, using resident cell types. Sustained drug delivery to the cornea was achieved and for voriconazole, the MIC against *Acanthamoeba castellanii* was attained alone and in combination with diclofenac. MTT and scratch wound data showed reasonable cell proliferation and wound repopulation at the drug doses used, supporting further development of the system to treat *Acanthamoeba* keratitis.

Keywords: *Acanthamoeba* keratitis, voriconazole, diclofenac, contact lenses

1. Introduction

Acanthamoeba keratitis (AK) is a serious, debilitating and potentially blinding inflammation of the cornea, caused by the infection of a free-living protozoan of the genus, *Acanthamoeba*. This amoeba resides in many environments including soil, air and water. The life cycle of *Acanthamoeba* includes two stages, an active trophozoite stage and a dormant cyst, both of which can be potentially pathogenic to humans (Clarke and Niederkorn, 2006). More than 80% of cases of this disease are found in contact lens wearers, since the wearing of contact lenses might facilitate the direct inoculation of the protozoan (*Acanthamoeba Keratitis Fact Sheet for Healthcare Professionals*, 2017). The adherence of the trophozoite to the corneal epithelium is essential for producing the infection. The pathogenic cascade of AK begins when trophozoites bind to mannose glycoproteins of the corneal epithelium through mannose-binding protein on the trophozoite membrane. After binding occurs, trophozoite-mediated destruction of the corneal epithelium commences via several mechanisms, such as direct cytolysis, apoptosis and phagocytosis. The pathogenic cascade proceeds with the penetration of the Bowman's membrane and the dissolution of the underlying collagenous stroma (Clarke and Niederkorn, 2006). An intense inflammation response is also a common sign of this pathology, with patients having very reddened eyes. In many cases, there is a loss of stromal keratocytes, infiltration of leukocytes, vascular congestion and chronic inflammation of the perilimbal bulbar conjunctiva (Garner, 1993). This amoeba also causes other symptoms, including foreign body sensation, decreased visual activity, photophobia and tearing. Early signs of AK may be mild and non-specific, with possible findings including epithelial irregularities, epithelial or stromal ulceration and infiltrates known as pseudodendrites. Later signs include stromal infiltrates, satellite lesions, radial keratoneuritis, scleritis and anterior uveitis. Advanced signs include stromal thinning and corneal perforation (Bernfeld et al., 2014).

To achieve successful treatment of AK, early diagnosis and aggressive medical therapies are critical. Currently, the treatment of this disease involves topical eye drop delivery of a combination of antimicrobial membrane-acting agents such as chlorhexidine (0.2%), which at minimal concentrations is not toxic to corneal epithelial cells (Lim et al., 2008; Ferrari et al., 2011, Itahashi et al., 2011), with polyhexamethylene biguanide (PHMB), which is effective at low concentrations (0.02%) but is unfortunately toxic to human corneal keratocytes (Lee et al., 2007), brolene or hexamidine. Eye drops containing these agents must be administered hourly during waking hours for 3 days; and then every 3 h for the following 3-4 weeks (Haburchak, 2017). However, 10% of the patients are resistant to treatment with chlorhexidine, in which case a combination of other

antimicrobial drugs should be used. The main drugs used are amphotericin B, rifampicin, pentamidine, flucytosine, pyrimethamine and cotrimoxazole. Even azole antifungal drugs, such as ketoconazole, voriconazole or clotrimazole, can be used orally or topically. The concomitant use of anti-inflammatory corticosteroids or NSAIDs is controversial and most authorities recommend it only when anterior-chamber inflammation is present. However, the biggest problem for the treatment of AK is the poor penetration of drugs into the stroma, the site of infection. Despite the easy accessibility of the eye for administering medications, the structure of the eye is very complicated, whilst the drugs used for the treatment of this disease are often molecules with high molecular weights and poor water solubility. This poses a significant challenge, in terms of their passage through the ocular barriers to the stroma. The main routes for administration of ophthalmic formulations are systemic, intravitreal and topical drug delivery. Systemic drug delivery needs high drug concentrations in the blood plasma, and this often exposes the patient to undesirable side effects. Instead, intraocular drug delivery by intravitreal injection is very effective in getting drugs to the posterior segment of the eye but is an invasive procedure with low patient compliance. Lastly, topical drug delivery is the most accepted route, as the solutions are relatively simply to formulate and there is high compliance from patients. However, traditional eyes drops are diluted in the tear film, eliminated quickly by the action of blinking and washed out by tears and nasolacrimal drainage. After instillation, only 1% or less of the drug reaches the target, the rest will be systemically adsorbed by the conjunctiva or nasolacrimal mucosa. Therapeutic contact lenses have been proposed as an ocular drug delivery mechanism that can overcome the limitations associated with conventional routes. Contact lenses are easy to administer and do not interfere with vision or normal eye functioning. They also offer controlled and sustained delivery of ocular drugs due to their unique properties of extended wear and more than 50% bioavailability in comparison to eye drop formulations (Li and Chauhan, 2006; Peng et al., 2010, 2012; González-Chomón et al., 2013). Release of drugs from therapeutic contact lenses occurs in the pre- and post-lens tear film, which leads to a residence time of more than 30 minutes, compared to just 1-3 minutes for eye drop formulations (Mcnamara et al., 1999; Creech et al., 2001). The high drug residence time increases the bioavailability up to 50%, which ultimately reduces the dose, dosing frequency, systemic drug absorption and its associated side effects (Jain, 1988; Li and Chauhan, 2006; Li and Chauhan, 2007; Xinming et al., 2008).

The drugs of choice in this work were voriconazole and diclofenac. Voriconazole is an antifungal drug that belongs to the triazole group. It acts by binding to the cytochrome P-450 enzyme lanosterol 14-α-demethylase, which is essential for the fungal cell membrane. It results in an alteration of the cell membrane and an enhanced permeability that causes cell dysfunction and a halt in growth

(Lin et al., 2013). Voriconazole is used for the treatment of invasive aspergillosis, for serious *Scedosporum* spp. or *Fusarium* spp. infections and for severe and fluconazole-resistant *Candida* spp. infections. It is also used for immunocompromised patients with life-threatening infections. This drug is administered via oral and intravenous routes; being available in the form of tablets, oral suspension and powder for solution infusion. Voriconazole is a lipophilic compound with low solubility (0.061% at pH 7), and is unstable in aqueous environments (Davies, 2000; Silveira, 2007). Whilst lipophilic compounds like voriconazole have higher corneal permeability, they usually have limited aqueous solubility. As such, formulating drug solutions can be challenging (Davies, 2000). For the formulation of a voriconazole drug solution to be feasible, the compound must be complexed with a β-cyclodextrin derivative. Cyclodextrins are a group of homologous cyclic oligosaccharides that, in complex formation with a drug, increase dissolution rate (solubility), aqueous stability, and/or bioavailability of the drug (Järvinen et al., 1995). This increases the solubility and stability of voriconazole in aqueous solutions, while maintaining its lipophilicity and high corneal permeability (Järvinen et al., 1995; Davies, 2000). The secondary drug, diclofenac sodium is the sodium salt of diclofenac, a non-steroidal anti-inflammatory agents (NSAID), with a mechanism of action involving non-selective reversible and competitive inhibition of the enzyme cyclooxygenase (COX). The blockade of COX inhibits the conversion of arachidonic acid into prostaglandins involved in pain, inflammation and fever. For this reason, it has been used for a long time in the treatment of diseases due to a downregulation of the inflammatory cascade. Diclofenac is currently indicated for inflammatory based eye conditions, usually used as 0.1% eye drops (*Diclofenac Sodium*, 2018). For the treatment of AK, it could be used in combination with an amoebicidal drug, such as voriconazole, to reduce pain and inflammation in the cornea.

Voriconazole has been investigated for its amoebicidal activity against *Acanthamoeba castellanii* and *Acanthamoeba polyphaga*, which are the most common species to cause keratitis (Dart et al., 2009). It was shown that it is a strong inhibitor of AcCYP51 activity and an effective inhibitor of trophozoite proliferation *in vitro* (Lamb et al., 2015). Recently, voriconazole has been successfully used both topically and systemically in human AK cases (Bang et al., 2010; Tu et al., 2010; Arnalich-Montiel et al., 2012; Cabello-Vilchez et al., 2014). It has been reported in a case report that 1% topical voriconazole was effective in the treatment of two in three eyes affected by AK, but resistant to chlorhexidine treatment (Bang et al., 2010). Although voriconazole can be considered a strong candidate for the treatment of human AK, it has been found in a study designed to investigate rat cornea penetration of eye drop and oral voriconazole that drug concentrations were directly dependent on the frequency of eye drop instillations, which resulted in lower plasma concentrations, whilst oral

voriconazole resulted in lower cornea concentrations (Gueudry et al., 2018). Also, despite voriconazole being inhibitory on clinical isolates of *Acanthamoeba*, amoebae have shown recovery from the effects of the drug upon transfer to a drug-free medium after a week or more (Schuster et al., 2006; Visvesvara et al., 2007). The results of these studies underline the need for high voriconazole corneal concentrations for efficient AK therapy, and the requirement for alternative routes of administration that achieve these concentrations whilst avoiding the use of high-frequency eye drop instillation regimens and high systemic doses of voriconazole.

This study aimed to test the plausibility of topically delivering voriconazole and voriconazole plus diclofenac to the cornea using drug-loaded, hydrogel contact lenses and determine to what extent the drugs were released and delivered to the cornea *in vitro* compared to equivalent concentration eye drop formulations.

2. Materials and methods

2.1. Materials

Voriconazole was purchased from Acros Organics (Geel, Belgium). 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CD) was purchased from Alfa Aesar (Poole, UK). HPLC grade water, HPLC grade methanol, HPLC grade acetonitrile, phosphate buffer saline (PBS) tablets, bovine serum albumin (BSA) and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Loughborough, UK). Diclofenac sodium salt, acetic acid, sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$) and sodium bicarbonate ($NaHCO_3$) were purchased from Sigma-Aldrich Company Ltd (Poole, UK). ACUVUE TruEye contact lenses (46% water, 54% narafilcon A) were purchased from Johnson & Johnson Vision Care (Limerick, Ireland). Freshly excised porcine eyes were obtained from a local abattoir. Human corneal epithelial cells (HCE-2 [50.B1] ATCC® CRL-11135) were purchased from LGC Standards (Middlesex, UK). Keratinocyte serum-free media (KSFM) and bovine collagen type I was purchased from Gibco, (Paisley, UK). Human corneal keratocytes (HK), poly-L-lysine and fibroblast (FB) media were purchased from ScienCell (San Diego, CA, USA). Human fibronectin was purchased from Roche Diagnostics (Mannheim, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Tocris/Bio-Techne (Bristol, UK).

2.2. Preparation of solutions

A 1% w/v voriconazole solution was made by adding 500 mg of voriconazole to 50 mL of distilled deionised water. 8 g of 2-HP-β-CD was then slowly added to the solution under constant stirring; an ultrasonic water bath was also used to aid drug dissolution. 'Eye drop' formulations, containing the same concentrations of voriconazole and diclofenac absorbed by the contact lenses were used as comparators (Section 2.3.). A simple electrolyte-based simulated tear fluid (STF) was made by dissolving 6.7 g NaCl, 2.2 g $NaHCO_3$, 0.61 g $CaCl_2$ and 1.4 g KCl in 1 L distilled deionised water.

2.3. Drug-loaded contact lenses

The 'breathing in' technique was used to prepare drug-loaded contact lenses, whereby dehydrated lenses were placed in drug solution and allowed to imbibe drug whilst re-establishing normal hydration level. Firstly, 1-day Acuvue TruEye contact lenses were soaked in 20 mL of distilled deionised water for 3-4 h on a Cole-Parmer STR9 Gyro Rocker at room temperature to remove salts. The lenses were then dried in an oven at 60 °C for 1 h to remove all the moisture, and then weighed. The dried lenses were put in 10 mL of a 1% w/v voriconazole solution or 1% w/v voriconazole and 0.5% w/v diclofenac and left overnight on a tube rotator at room temperature to allow rehydration and impregnation with the drug. The following day, lenses were carefully removed from the drug solution using forceps, gently dried on tissue paper and reweighed. The difference between the soaked weight and dried weight of the lenses is the amount of drug solution taken up, with the amount of drug absorbed by the lenses calculated based on the concentration of the solution of drug used.

2.4. Drug release profile from contact lenses

Hydrogel drug-loaded contact lenses were prepared, as described above. Each lens was put in a Falcon™ 15 mL centrifuge tube (Fisher Scientific UK Ltd, Loughborough, UK) containing 10 mL of distilled deionised water and left on a Cole-Parmer STR9 Gyro Rocker at room temperature. A 1 mL sample was taken after 10 min, 1 h, 2 h, 4 h and 6 h, and transferred to autosampler vials for analysis by HPLC, as described below. Following the 10 μL injection per time point, the 990 μL was retransferred to the Falcon™ 15 mL centrifuge tube.

2.5. Drug delivery into porcine eyes

Porcine eyes were collected on the day of slaughter from a local abattoir, stored at 4 °C overnight and then used for experimentation within 12 h. Before experimentation, eyes were examined for abrasion or scarring, and any excess muscle and fat was removed using scissors. The eyes were put with cornea uppermost in individual wells of a 6-well plate containing 1 mL PBS, to prevent dehydration, and placed in a 37 °C water bath for 10 min. Subsequently, drug-loaded, contact lenses or 50 µL of equivalent concentration eye drops were applied. To mimic tear fluid, STF was dripped onto each eye using a 50 mL syringe driver with 12 tubes attached to bathe each eye, at a rate of 20 mL/h to simulate steady tear flow (Hewitt et al., in press). Eyes were left in the water bath for 2 h, 4 h or 6 h, to determine the kinetic release from the system and the penetration of the drug into the cornea.
After each experiment, the eyes were taken out of the water bath and the contact lenses were removed. Using a scalpel and surgical scissors, each cornea was dissected and then extracted twice in methanol. Each cornea was placed in a 2 mL microcentrifuge tube with 1 mL of HPLC grade methanol, and put on a Fisher Scientific multi-purpose tube rotator at room temperature overnight. The following day each cornea was removed and placed in a second 2 mL microcentrifuge tube with 1 mL of fresh HPLC grade methanol; the previous methanol fraction was preserved in the fridge. The microcentrifuge tubes containing corneas were then put back on to the tube rotator again overnight. The following day the two methanol fractions were reunited into one microcentrifuge tube and left to evaporate overnight in a 60 °C oven. The methanol from both extraction steps were combined, centrifuged (3500 RPM) for 15 min and supernatants evaporated at 60 ºC overnight. The resulting residue was reconstituted with 1 mL fresh HPLC grade methanol and transferred to autosampler vials for HPLC.

2.6. HPLC analysis

HPLC was used to quantify drug release from the contact lenses and the amounts reaching the corneal stroma. For the quantitative analyses, an Agilent 1100 instrument fitted with a Phenomenex Kinetex 5 µm C18 150 x 4.6 mm column was used. For voriconazole detection, a mobile phase composed of 50% water and 50% acetonitrile was prepared. The UV detector was set at 256 nm, the injection volume was 10 µL, the flow rate was 1 mL/min and the run time was 6 min. A calibration curve was produced using standard solutions of voriconazole through a concentration range of 1.95-1000 µg/mL; and the mean was $R^2 = 0.9999$. The retention time for the calibration curve and release profile was around 2.6 min, but with the corneas it was shifted to 1.55 min. For diclofenac detection, a mobile phase composed of 72% acetonitrile and 28% water was prepared, with the solution pH adjusted to 3.5 using acetic acid. The UV detector was set at 280 nm, the injection volume was 10 µL, the flow

rate was 1 mL/min and the run time was 5 min. The retention time was 1.9 min under these conditions. A calibration curve was produced using standard solutions of diclofenac through a concentration range of 1.95-1000 µg/mL and the mean was $R^2 = 0.9992$.

2.7. Minimum inhibitory concentration (MIC)

The MIC for voriconazole against *Acanthamoeba castellanii* was determined as described previously (Lamb et al., 2015) and found to be 0.5 µg/mL, which is reduced to 0.25 µg/mL when diclofenac is at 16 µg/mL or higher. For diclofenac, the MIC against *Acanthamoeba castellanii* is 128.0 µg/mL. All the data obtained in this study were expressed in average µg/cornea, for comparing with the MIC value they were converted to concentration values (µg/mL) using the following equation (Equation 1):

$$Conc\left({}^{\mu g}/_{mL}\right) = \frac{\mu g\ per\ cornea}{Volume_{cornea}}$$

The volume of a standard porcine cornea was determined by measuring the wet and dry weights of porcine corneas (n=6) and then calculating the average total mass of water (g) within each specimen (wet weight (g) – dry weight (g) = total g of water). The total mass of water (g/cornea) is the equivalent of the amount of mL per cornea (mL/cornea), which was used in the equation above (Equation 1) for corneal volume.

2.8. Cell viability evaluation

An MTT assay was performed to assess the cytotoxicity of the voriconazole and/or diclofenac concentrations found by HPLC to be localized in porcine corneas 2 h, 4 h and 6 h post-application of the drug-loaded, contact lenses. Coated flat-bottomed, polystyrene 96-well plates were seeded with either 8,000 HCE-2 in 100 µL supplemented KSFM (well plates coated with 0.01 mg/mL BSA, 0.01 mg/mL fibronectin and 0.03 mg/mL collagen type I in PBS) or 8,000 HK in 100 µL supplemented FB media (well plates coated with 2 µg/cm^2 poly-$_L$-lysine). Prepared plates were maintained at 37 °C in a humidified 5% CO_2/95% air atmosphere for 24 h. Exposure to the test agents was initiated by adding the concentration of voriconazole and/or diclofenac (µg/mL) that corresponded to the amounts found to be localized following the use of contact lenses to three wells containing HCE-2/HK and three cell-free wells (per single or combination treatment) at the longest time-point (6 h exposure). This was repeated for the corresponding concentrations at each time point. For the untreated control wells, 10 µL of sterile HPLC grade water was added. Aliquots of MTT stock solution, previously

prepared in PBS and frozen at -20 °C, were thawed while protected from light. MTT stock solution was diluted in an uncoated 25 cm^2 culture flask 1:5 with supplemented KSFM to a final concentration of 1 mg/mL and placed in the incubator to warm and equilibrate for 30-45 min before use. The relative number of viable cells was determined by adding diluted MTT solution for 2 h, followed by DMSO for 30 min. A Tecan Infinite M200 Pro Microplate Reader was used to measure the absorbance in each well at 506 nm, the λ_{max} for the MTT formazan product formed, which was subsequently solubilized in 100 % DMSO.

In order to interpret the raw MTT assay data, the absorbance readings of the cell-free wells (blanks) were firstly subtracted from each of their treated HCE-2/HK containing equivalents. Mean values for each technical triplicate were then calculated and the viability as a percentage of untreated controls were determined for each concentration of voriconazole and/or diclofenac using the following equation (Equation 2):

$$\%\, Viability = \frac{[Formazan\ absorbance\ from\ treated\ cells]}{[Formazan\ absorbance\ from\ treatment-free\ controls]} \times 100$$

2.9. Scratch wound assay using corneal epithelial cells

The rate of wound closure *in vitro* was determined using Automated Confocal Time-Lapse Microscopy, based on the method of Hardwicke et al. (2010). HCE-2 cells were seeded into 24-well plates in KSFM and maintained at 37 °C in a humidified 5% CO_2/95% air atmosphere, until confluent. Confluent HCE-2 monolayer cultures were scraped in a straight line with sterile p200 pipette tips to create a “scratch” wound, washed (x2) with PBS to remove debris and the KSFM replaced with treatment media containing the appropriate concentrations of voriconazole and/or diclofenac. Cultures were maintained at 37 °C in a humidified 5% CO_2/95% air atmosphere, with the migration of cells into the denuded area monitored using the Leica TCS SP5 Confocal Microscope (Leica Microsystems, Milton Keynes, UK). Digital images were taken every 20 min over 6 h and converted to videos using LAS X Software (Leica Microsystems, Milton Keynes, UK). HCE-2 scratch wound repopulation rates were quantified using ImageJ® Software (Version 1.49, *https://imagej.nih.gov/ij/*), with data expressed as percentage wound closure at 2 h, 4 h and 6 h, versus wound areas at 0 h. Percentage wound closure rates were subsequently compared versus those of corresponding untreated control cultures.

2.10. Data analysis

The data were analysed using Excel 2016 (Microsoft Office, Microsoft Corp., Redmon, WA, USA) and expressed as a mean ± SEM. Statistical analysis was carried out with InStat® for Macintosh, version 3.00 (GraphPad Software Inc., San Diego, CA, USA). The confidence interval was 95% and $p < 0.05$ was considered to be significant.

3. Results and Discussion

AK is an increasingly widespread corneal disease of infectious origin caused by a free-living protozoan of the genus *Acanthamoeba* (Thebpatiphat et al., 2007). Although contact lens wear is the principle risk factor for the disease (Ibrahim et al., 2009), *Acanthamoeba* can also cause infection in non-contact lens wearers. A delay in diagnosing and treating AK can lead to significant loss of vision due to the destruction caused by the presence of the microorganism (Bacon et al., 1993). As *Acanthamoeba* infection increases in incidence, new therapies are required to overcome organisms that have become resistant to commonplace treatments. Furthermore, there are obstacles with ocular administration of medication, considering the physiological barriers of the eye, the unwanted side effects of systemic delivery and the invasive nature of intraocular methods. This study investigated the *ex vivo* use of anti-amoebic drug-loaded hydrogel contact lenses containing voriconazole alone or in combination with anti-inflammatory diclofenac on porcine corneas for the treatment of AK.

The simplest way to load a drug into soft contact lenses is to soak preformed lenses in a solution of the drug. This technique relies on the passive diffusion of drug molecules into the lens matrix, but has the disadvantage of slow attainment of equilibrium, whereas the ‘breathing-in’ technique allows for much more rapid drug loading following lens rehydration (unpublished data).

3.1. Drug release profile from contact lenses

The percentage cumulative drug release profiles for the release of the drug(s) from single and binary-loaded contact lenses were created in order to understand the kinetic release-rate of the drugs from the vehicle (Figures 1 and 2). An ideal kinetic release would be a zero-order model. Zero-order release kinetics describe systems where the drug release rate is constant over a period of time (Ciolino et al., 2009). This allows the necessary concentration to be achieved quickly and be maintained throughout treatment duration. After ‘breathing-in’ 1% w/v voriconazole solution, hydrogel contact lenses absorbed 153.7 ± 6.7 µg voriconazole. The drug release profile (Figure 1) revealed that an average of 14.0 ± 0.3 µg voriconazole was released after 10 min (9.1%), 21.1 ± 3.4 µg (22.9%) after 1 h, 23.4 ±

0.4 µg (38.1%) after 2 h, 24.7 ± 1.9 µg (54.2%) after 4 h; and 27.1 ± 2.7 µg (71.8%) after 6 h. When the hydrogel contact lenses 'breathed in' 1% w/v voriconazole and 0.5% w/v diclofenac solution, they absorbed 173.7 ± 17.9 µg voriconazole and 86.8 ± 8.9 µg diclofenac per contact lens. The drug release profile (Figure 2) revealed that after 10 min, an average of 4.1 ± 0.1 µg (2.3%) voriconazole and 10.6 ± 0.6 µg (12.3%) diclofenac were released, an average of 5.5 ± 0.7 µg (5.5%) voriconazole and 17.3 ± 1.4 µg (32.1%) diclofenac after 1 h, 7.5 ± 0.5 µg (9.8%) voriconazole and 19.0 ± 0.6 µg (54.0%) diclofenac after 2 h, 13.8 ± 1.4 µg (17.8%) voriconazole and 20.2 ± 0.6 µg (77.3%) diclofenac after 4 h; and an average of 19.3 ± 1.1 µg (28.9%) voriconazole and 17.8 ± 3.6 µg (97.8%) diclofenac after 6 h. For 1% w/v voriconazole-loaded contact lenses, there was a steady increase in drug release. Voriconazole is a lipophilic drug and the formulation required the inclusion of 2-HP-β-CD as a solubilizer. The low water solubility and the presence of the cyclodextrin facilitate continuous, prolonged delivery of the drug to the cornea, without resulting in peaks of high drug concentration. The release of voriconazole from binary drug-loaded contact lenses was more gradual and resulted in lower drug release at each time point due to steric hinderance occurring between the voriconazole/2-HP-β-CD complex and the secondary drug, diclofenac. The binary loading of voriconazole/2-HP-β-CD and diclofenac could result in increased steric bulk within the confines of the polymeric network, resulting in slower drug release. The release of diclofenac from the binary contact lenses was greater but still steady, obtaining a release percentage of 97.8% by 6 h.

3.2. Comparing 1% w/v voriconazole-loaded contact lenses with equivalent concentration eye drops

1% w/v voriconazole-loaded contact lenses were compared to 50 µL of an equivalent concentration eye drop formulation (Figure 3, Tables 1 and 2). After 'breathing in' 1% w/v voriconazole solution, the contact lenses absorbed an average of 157.7 ± 10.5 µg (n=9 ± SEM) voriconazole. The amount of drug delivered to the cornea was calculated as a percentage of the amount administered (n=3 contact lenses) for the three corneas at each time point. After 2 h, the contact lenses delivered an average of 12.7 ± 0.3 µg (n=3 ± SEM) voriconazole to the cornea, equivalent to an average of 9.0 ± 1.8%. After 4 h, the lenses delivered an average of 14.7 ± 1.1 µg (n=3 ± SEM) of drug per cornea, equivalent to an average of 8.3 ± 1.0% of the amount administered. Then, after 6 h, the lenses delivered an average of 7.3 ± 0.3 µg (n=3 ± SEM) voriconazole for each cornea, equal to an average of 5.2 ± 0.4% of the quantity absorbed. As a comparison, 50 µL of eye drop formulation containing 155 µg voriconazole was used. Initially, after 2 h, the eye drops delivered an average of 6.0 ± 1.5 µg (n=3 ± SEM) of drug, equivalent to an average of 3.9 ± 1.0% of the amount administered. After 4 h, the eye drops delivered an average of 7.1 ± 1.1 µg (n=3 ± SEM) voriconazole, equal to an average of

4.6 ± 0.7% of the amount in the formulation. Then, after 6 h, equivalent concentration eye drops delivered an average of 5.3 ± 0.3 µg (n=3 ± SEM) voriconazole per cornea, equivalent to an average of 3.4 ± 0.2% of the amount administered. The concentration of voriconazole found to localise within the cornea following the application of voriconazole-only loaded contact lenses ranged between 38.5 and 77.6 µg/ml through the time points. The concentration of voriconazole found to localise within the cornea following the application of voriconazole-only eye drops ranged between 27.8 and 37.4 µg/ml through the time points. These concentration values align well with values found by a previous study conducted on rabbit corneas (Sun et al., 2008), which found that the drug concentration in the cornea following a single 50 µL dose of 1% voriconazole peaked at an average of 40 µg/ml. Therefore, 1% w/v voriconazole-loaded, contact lenses delivered more voriconazole than equivalent concentration eye drops at each time-point, with these differences being significant at 2 h (p <0.001) and 4 h (p <0.001) post-treatment.

3.3. Comparing 1% w/v voriconazole and 0.5% w/v diclofenac-loaded contact lenses with equivalent concentration eye drops

1% w/v voriconazole and 0.5% w/v diclofenac-loaded contact lenses were compared to 50 µL of an equivalent concentration eye drop formulation (Figure 4, Tables 1 and 2). When allowed to 'breathe in' the solution overnight, the contact lenses absorbed an average of 189.9 ± 6.1 µg (n=9 ± SEM) voriconazole and 94.9 ± 3.1 µg (n=9 ± SEM) diclofenac. After 2 h, the contact lenses delivered an average of 11.7 ± 1.2 µg (n=3 ± SEM) voriconazole and 23.0 ± 5.1 µg (n=3 ± SEM) diclofenac per cornea. After 4 h, the lenses delivered an average of 15.4 ± 3.9 µg (n=3 ± SEM) voriconazole and 27.8 ± 4.3 µg (n=3 ± SEM) diclofenac per cornea. Then, after 6 h, the lenses delivered an average of 11.7 ± 1.8 µg (n=3 ± SEM) voriconazole and 23.4 ± 0.9 µg (n=3 ± SEM) diclofenac for each cornea. The amount of both drugs delivered to the cornea was again calculated as a percentage of the amount administered (n=3 contact lenses) for the three corneas at each time point. An average of 6.5 ± 0.8% voriconazole and 12.9 ± 1.6% diclofenac were delivered after 2 h from loaded contact lenses. After 4 h, the lenses delivered an average of 8.1 ± 1.8% voriconazole and 16.1 ± 3.6% diclofenac of the amounts absorbed. Lastly, after 6 h, the contact lenses delivered an average of 6.0 ± 0.9% voriconazole and 12.0 ± 1.8% diclofenac of the amounts absorbed. After 2 h, equivalent concentration eye drops delivered an average of 3.4 ± 1.1 µg (n=3 ± SEM) voriconazole and 5.3 ± 1.7 µg (n=3 ± SEM) diclofenac per cornea. After 4 h, the eye drops delivered an average of 6.6 ± 1.4 µg (n=3 ± SEM) voriconazole and 4.9 ± 0.4 µg (n=3 ± SEM) diclofenac per cornea. Lastly, after 6 h, equivalent concentration eye drops delivered an average of 5.9 ± 1.1 µg (n=3 ± SEM) voriconazole and 4.9 ±

0.8 μg (n=3 ± SEM) diclofenac for each cornea. After 2 h, an average of 2.2 ± 0.7% voriconazole and 3.0 ± 0.9% diclofenac of the amounts present in the solution were delivered. After 4 h, an average of 4.3 ± 0.9% voriconazole and 2.7 ± 0.2% diclofenac were delivered to the cornea. Then, after 6 hours an average of 3.8% ± 0.7% of voriconazole and 2.7 ± 0.4% diclofenac were delivered.

The concentration of voriconazole found to localise within the cornea following the application of binary-loaded contact lenses ranged between 61.7 and 81.3 μg/ml through the time points. The concentration of voriconazole found to localise within the cornea following the application of equivalent concentration eye drops ranged between 17.7 and 35.0 μg/ml through the time points. As with the single drug-loaded contact lenses, binary drug-loaded contact lenses delivered more voriconazole than equivalent concentration eye drops at each time-point and the localised concentration of voriconazole following the application of equivalent concentration eye drops corroborates well with values found in previous studies (Sun et al., 2008). The differences between binary contact lenses and equivalent concentration eye drops were significant at 2 h (p <0.05), 4 h (p <0.01) and 6 h (p <0.05) post-treatment. Performance was further improved by loaded contact lenses compared to eye drops for the delivery of diclofenac, with significant differences at 2 h (p <0.01), 4 h (p <0.01) and 6 h (p <0.01) post-treatment. The delivery of diclofenac was consistently lower in combination than when delivered alone.

3.4. Effect of voriconazole and/or diclofenac on corneal keratocyte and epithelial cell survival

To address whether the concentrations of voriconazole and/or diclofenac that localised within the stroma influenced resident cell survival, an MTT assay was used to examine cell viability. En route to the stroma, the delivered drugs would encounter both epithelial cells and stromal keratocytes. HCE-2 and HK cells were treated with the appropriate concentrations of voriconazole and/or diclofenac in KSFM/FB media for each corresponding exposure time (Table 3). HCE-2 cell viability was shown to remain above 90% compared to untreated controls when exposed to voriconazole alone, whether for short (2 h, 67.1 μg/mL) or longer periods (6 h, 38.47 μg/mL). However, exposure to diclofenac alone appeared to have more of an impact on cell viability, with HCE-2 viability decreasing from 82.7% after 2 h exposure (289.95 μg/mL) to 62.4% after 6 h exposure (369.47 μg/mL). HK viability was shown to remain above the viability of the untreated controls when exposed to voriconazole alone, whether for short (2 h, 67.1 μg/mL) or longer periods (6 h, 38.47 μg/mL). In fact, voriconazole appeared to have a positive effect on the cells, with higher drug concentrations resulting in greater HK viability than at lower concentrations, irrespective of exposure time. However, as with the HCE-

2 cells, exposure to diclofenac alone appeared to have more of an impact on cell viability, with HK viability decreasing from 95.5% after 2 h exposure (289.95 μg/mL) to 91.7% after 6 h exposure (369.47 μg/mL). HCE-2 viability when voriconazole and diclofenac were used in combination ranged between 85.6-72.8%. HK viability when the drugs were used in combination decreased from above the viability of the untreated controls after 2 h exposure (61.84 μg/mL voriconazole and 121.1 μg/mL diclofenac) to 92% after 6 h exposure (61.68 μg/mL voriconazole and 123.32 μg/mL diclofenac). Both cell types maintained relatively high levels of viability when exposed to corresponding concentrations of voriconazole alone at all three time-points assessed (greater than 90%). This was particularly the case with the keratocytes, which were especially resistant to the induction of any cytotoxic effects. Previous work has shown that voriconazole below concentrations of 25 μg/mL has no cytotoxic effect on corneal epithelial cells after an exposure time of 24 h (Sobolewska et al., 2015). In the current work, epithelial cells were exposed to higher concentrations (up to ~78 μg/mL), but only for a quarter of the time. After 24 h, the equivalent concentration (within a range of 50-100 μg/mL) resulted in percentage viability values between 73.8% (50 μg/mL) and 59.2% (100 μg/mL), compared to untreated controls (Sobolewska et al., 2015). With the use of shorter exposure times (<24 h) and at the localised drug concentrations, it is unlikely that the epithelial cells would experience any significant detrimental effects from voriconazole alone. Diclofenac displayed more cytotoxicity on both cell types, whether alone or in combination with voriconazole, again to a greater extent than epithelial cells. Lower concentrations of diclofenac were applied when in combination, as less of the drug was found to localise in the cornea under these conditions. However, the presence of diclofenac still had an impact on cell viability to some degree.

3.5. Effect of voriconazole and/or diclofenac on corneal epithelial wound healing in vitro

Early signs of AK include epithelial ulceration, thus a scratch wound repopulation assay was performed using a Leica TCS SP5 Confocal Microscope (Figure 5) to investigate the effects of the drugs on epithelial cell migration and repair. The localized concentrations of voriconazole alone significantly diminished the rates of wound closure by HCE-2 cells (3.8- and 2.3-fold respectively, Figure 5) compared to untreated controls, at 4 h (p <0.05) and 6 h (p <0.05). However, no significant differences in wound closure rates were identified between untreated and voriconazole-treated HCE-2 cells at 2 h post-treatment (p >0.05). The localised concentrations of diclofenac alone also significantly diminished wound closure rates by HCE-2 cells (2.0-, 4.3- and 84.4-fold respectively, Figure 5) versus untreated controls, at 2 h (p <0.05), 4 h (p <0.001) and 6 h (p <0.001). Also, the localized concentrations of the drugs in combination significantly diminished the rates of wound

closure by HCE-2 cells (2.9-, 11.3- and 26.0-fold respectively, Figure 5) compared to untreated controls, at 2 h (p <0.05), 4 h (p <0.001) and 6 h (p <0.001). The cells failed to migrate into the wound at the same rate as untreated controls overall, which implies that the drugs inhibited the migration and proliferation of treated cells. Again, the effects on the cells were greater in the presence of diclofenac. These results suggest that although diclofenac has the effect of blocking the inflammatory cascade, this is at the cost of somewhat decreasing cellular viability and their reparative capabilities. Previous work has reported on the toxicity of diclofenac sodium, with the drug being linked to toxicity in renal cortex mitochondria (Uyemura et al., 1997), NSAID-associated keratolysis (O'Brien et al., 2001) and corneal melting after LASIK (Hsu et al., 2003). In addition, NSAIDs inhibit COX activity in the arachidonic acid cascade and diminish prostaglandin synthesis. Prostaglandins are necessary for protein and DNA synthesis in epithelial cells. Therefore, use of NSAIDs (particularly diclofenac sodium) may affect corneal epithelial wound healing (Hersh et al., 1990; Lindstrom, 2006). Diclofenac sodium has also been reported as causing significant delays in early wound healing in the scraped rabbit corneal epithelium and re-epithelialisation after penetrating keratoplasty (Hersh et al., 1990). These previous findings could explain the impact on cellular viability and wound repopulation observed in this study. Nevertheless, it is important to note that the MIC for voriconazole necessary to inhibit visible growth of the infection, was achieved and exceeded using the drug-loaded, contact lenses, both when single- and binary-loaded at all time-points examined; whilst maintaining the viability and to a lesser extent, the functionality of resident cell types.

Overall, the drug-loaded, contact lenses proved superior to equivalent concentration eye drops and were shown to obtain consistent and sustained drug delivery to the cornea. For voriconazole, the MIC against *Acanthamoeba castellanii* was attained and surpassed, both alone and in combination with diclofenac throughout the study period. The delivery methods described here were tested *ex vivo* on porcine eyes, with constant dropwise irrigation of the eye with STF to account for tear production and to mimic the natural environment of the eye *in vivo* (Hewitt et al., in press). However, the pathologic condition of AK can present many adverse changes in the eye that could influence drug delivery. The epithelium is often damaged by the parasite through ulceration, immune cells can be present at the surface; and pH and temperature can also differ as a result of the inflammatory response. These variations in conditions could modify the permeation of the drug through the corneal tissue and influence the concentration of the localised drug into the corneal stroma.

4. Conclusions

The findings of this work indicate that topically delivered voriconazole via binary drug-loaded, hydrogel contact lenses is a plausible improvement upon other delivery methods for the treatment of AK, in terms of drug transport into the cornea and healing.

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Legends to Figures

Figure 1. Percentage cumulative drug release profile from 1% w/v voriconazole-loaded, contact lenses (n=3 ± SD)

Figure 2. Percentage cumulative drug release profile from 1% w/v voriconazole and 0.5% w/v diclofenac-loaded, contact lenses (n=3 ± SD).

Figure 3. Quantities of voriconazole delivered to the cornea by 1% w/v voriconazole-loaded, contact lenses and equivalent concentration eye drops after 2 h, 4 h and 6 h (n=3 ± SEM, *** = p <0.001).

Figure 4. Quantities of voriconazole and diclofenac delivered to the cornea by 1% w/v voriconazole and 0.5% w/v diclofenac-loaded, contact lenses and equivalent concentration eye drops after 2 h, 4 h and 6 h (n=3 ± SEM, * = p <0.05, ** = p <0.01).

Figure 5. Percentage wound closure rates for untreated, voriconazole and diclofenac-treated, HCE-2 cells for 2 h, 4 h and 6 h, at the corresponding localisation concentrations. (***** = p <0.05, *** = p <0.001).

Fig 1

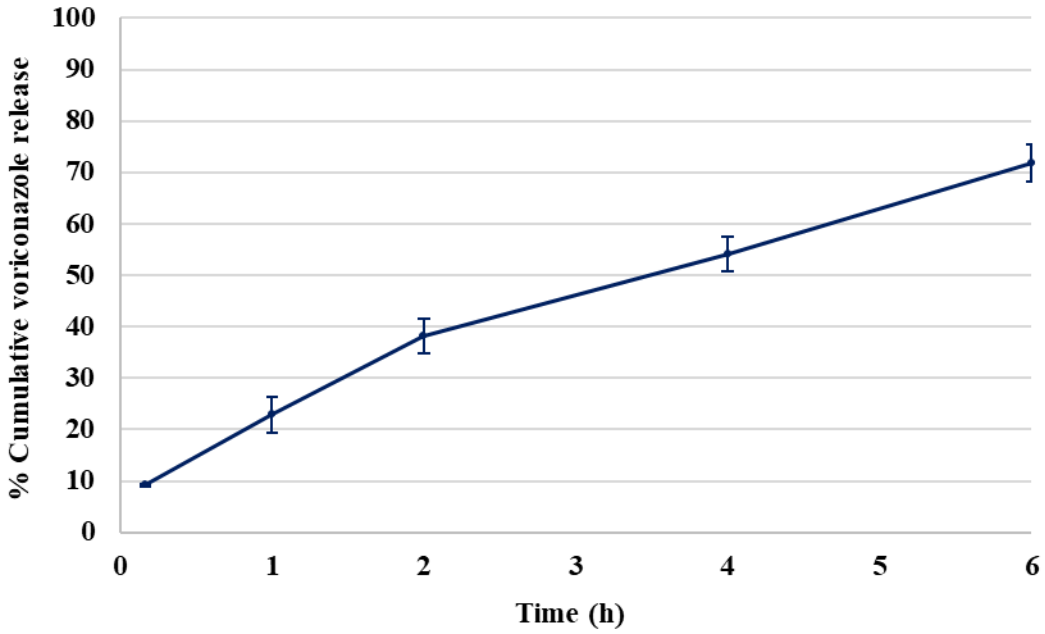
% Cumulative voriconazole release
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
Time (h)

Fig. 2

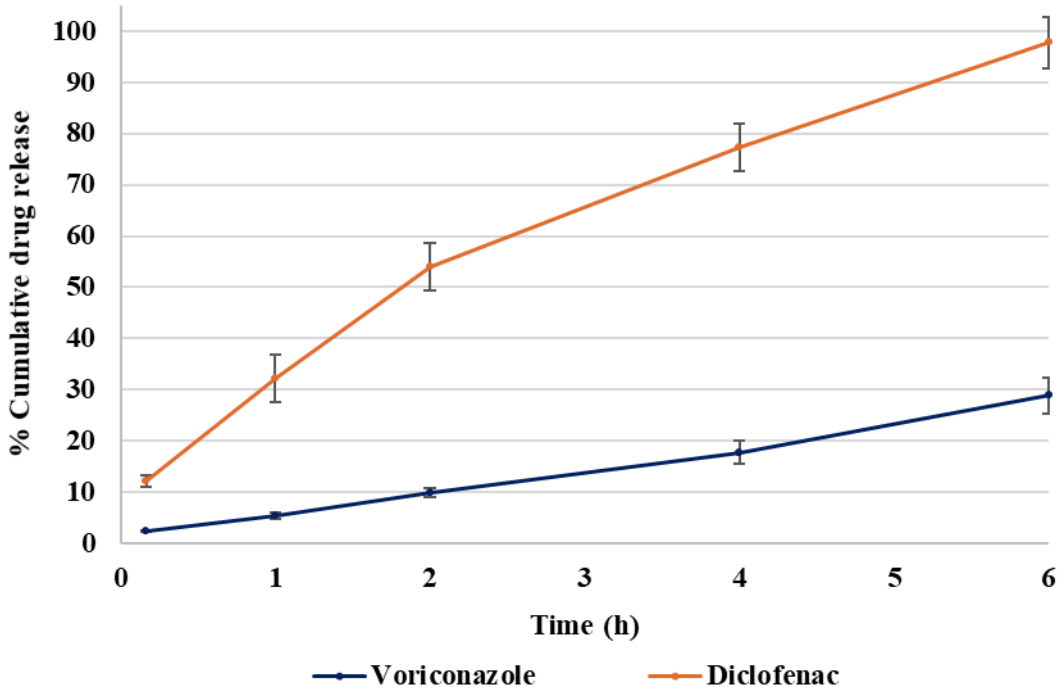
% Cumulative drug release
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
Time (h)
Voriconazole
Diclofenac

Fig 3

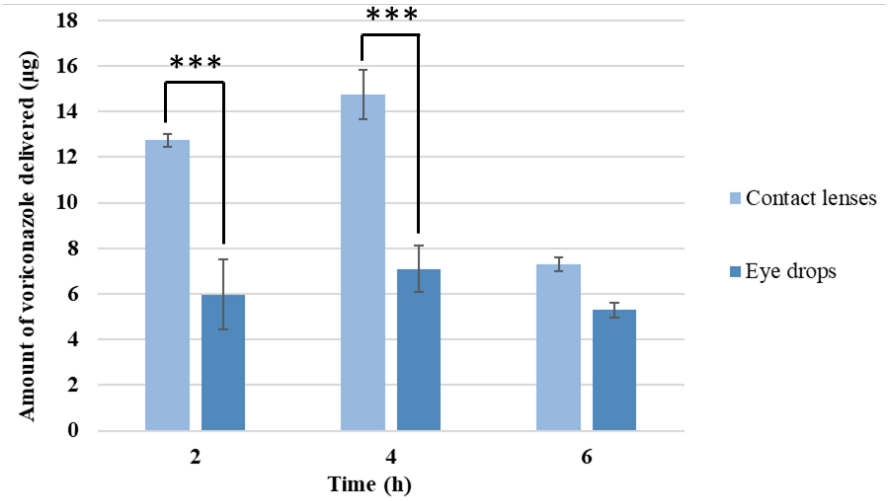
***
***
18
16
14
12
10
8
6
4
2
0
Amount of voriconazole delivered (µg)
2
4
6
Time (h)
Contact lenses
Eye drops

Fig 4

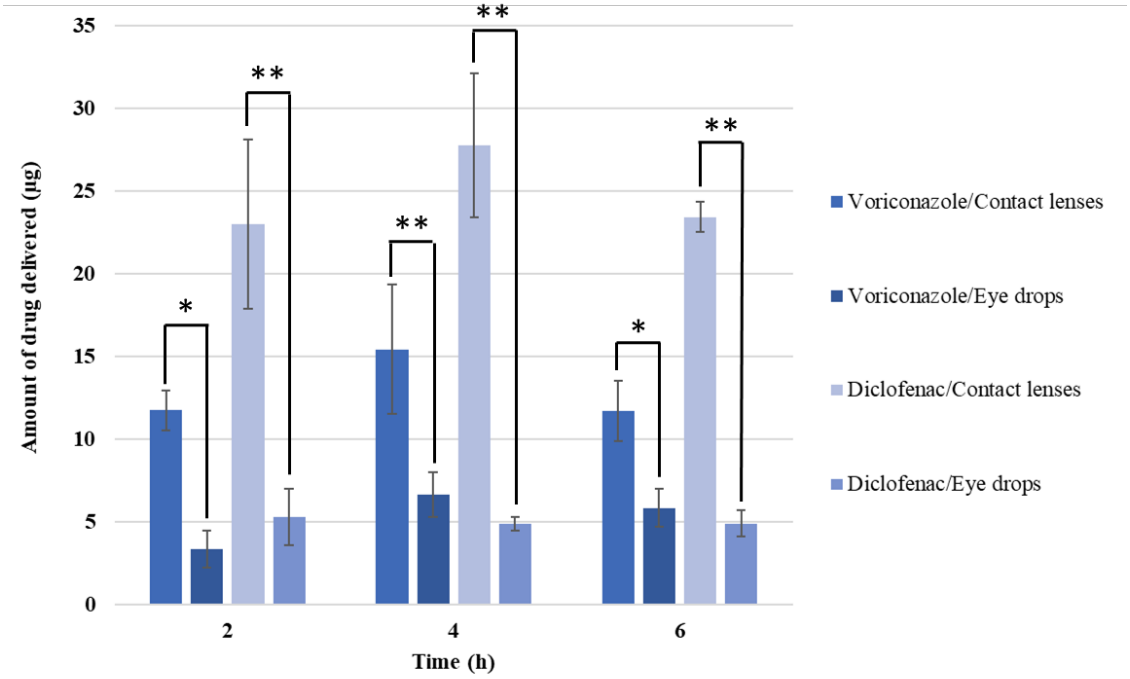
Amount of drug delivered (µg)
35
30
25
20
15
10
5
0
2
4
6
Time (h)
*
**
**
**
*
**
Voriconazole/Contact lenses
Voriconazole/Eye drops
Diclofenac/Contact lenses
Diclofenac/Eye drops

Fig 5

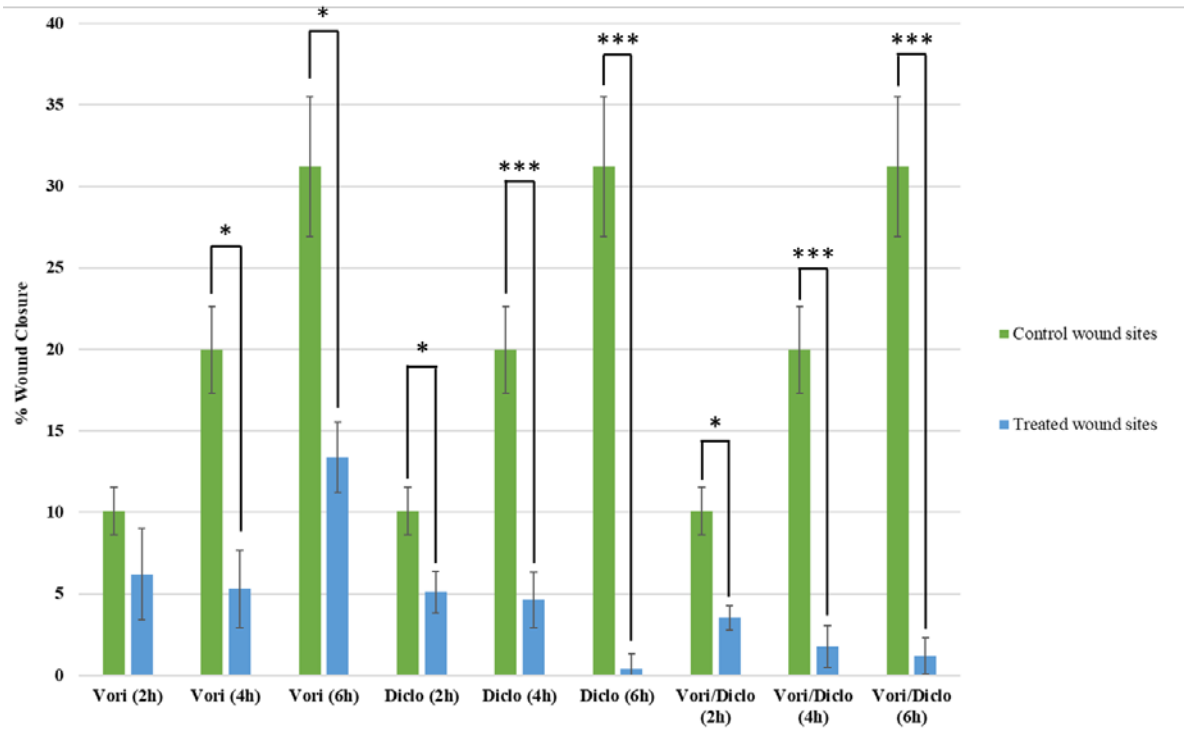
% Wound Closure
40
35
30
25
20
15
10
5
0
*
*
*
***
***
*
***
***
Vori (2h)
Vori (4h)
Vori (6h)
Diclo (2h)
Diclo (4h)
Diclo (6h)
Vori/Diclo (2h)
Vori/Diclo (4h)
Vori/Diclo (6h)
Control wound sites
Treated wound sites

Table 1. 1% w/v voriconazole and/or 0.5% w/v diclofenac-loaded contact lenses. The amount of drug found to localise in the cornea (µg/cornea) was converted to concentrations (µg/mL), in order to compare with the MIC values.

Sample	µg/cornea	Concentration in cornea (µg/mL)	MIC (µg/mL)
Voriconazole (2 h)	12.7	67.0	0.5
Voriconazole (4 h)	14.7	77.6	
Voriconazole (6 h)	7.3	38.5	
Diclofenac (2 h)	55.1	289.9	128.0
Diclofenac (4 h)	55.8	293.6	
Diclofenac (6 h)	70.2	369.5	
Voriconazole in combination (2 h)	11.8	61.8	0.25
Voriconazole in combination (4 h)	15.4	81.3	
Voriconazole in combination (6 h)	11.7	61.7	
Diclofenac in combination (2 h)	23.0	121.1	128.0
Diclofenac in combination (4 h)	27.8	146.1	
Diclofenac in combination (6 h)	23.4	123.3	

Table 2. 1% w/v voriconazole and/or 0.5% w/v diclofenac eye drop formulations. The amount of drug found to localise in the cornea (µg/cornea) was converted to concentrations (µg/mL), in order to compare with the MIC values.

Sample	µg/cornea	Concentration in cornea (µg/mL)	MIC (µg/mL)
Voriconazole (2 h)	6.0	31.5	0.5
Voriconazole (4 h)	7.1	37.4	
Voriconazole (6 h)	5.3	27.8	
Diclofenac (2 h)	23.4	123.0	128.0
Diclofenac (4 h)	15.9	83.8	
Diclofenac (6 h)	10.5	55.1	
Voriconazole in combination (2 h)	3.4	17.7	0.25
Voriconazole in combination (4 h)	6.6	35.0	
Voriconazole in combination (6 h)	5.9	30.8	
Diclofenac in combination (2 h)	5.3	28.0	128.0
Diclofenac in combination (4 h)	4.9	25.8	
Diclofenac in combination (6 h)	4.9	25.9	

Table 3. Concentrations of voriconazole and/or diclofenac localised in porcine corneas, as a function of drug exposure times and cell viability values.

Treatment (+ exposure time)	Concentration (µg/mL)	% Viability (HK)	% Viability (HCE-2)
Voriconazole (2 h)	67. 0	106.7	95.4
Voriconazole (4 h)	77.6	109.1	90.9
Voriconazole (6 h)	38.5	102.2	94.7
Diclofenac (2 h)	289.9	95.5	82.7
Diclofenac (4 h)	293.6	93.7	74.1
Diclofenac (6 h)	369.5	91.7	62.4
Voriconazole in combination (2 h)	61.8	108.2	85.6
Voriconazole in combination (4 h)	81.3	99.0	72.8
Voriconazole in combination (6 h)	61.7	92.0	77.4
Diclofenac in combination (2 h)	121.1	108.2	85.6
Diclofenac in combination (4 h)	146.1	99.0	72.8
Diclofenac in combination (6 h)	123.3	92.0	77.4